AD_____

Award Number: DAMD17-98-1-8021

TITLE: Role of cdc25 Phosphatases in Cellular Immortalization

PRINCIPAL INVESTIGATOR: Konstantin I. Galaktionov, Ph.D.

CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston, Texas 77030

REPORT DATE: April 2001

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	April 2001	Final (23 Mar 98 - 22 Mar 01)

4. TITLE AND SUBTITLE
Role of cdc25 Phosphatases in Cellular Immortalization

5. FUNDING NUMBERS
DAMD17-98-1-8021

6. AUTHOR(S)
Konstantin I. Galaktionov, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Baylor College of Medicine
Houston, Texas 77030

E-Mail: kgalakt@bcm.tmc.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

The goal of this proposal is to study the potential role of tyrosine phosphorylation-dependent checkpoint and especially the cdc25 phosphatase family of cyclin dependent kinase (CDK) activators in cellular immortalization. We have shown that cdc25 phosphatases did in fact extend the normal mammary epithelial cells (HMEC) life span, but did not immortalize them. The effect was most prominent with cdc25A and cdc25B phosphatases. Cdc25C phosphatase had less effect on HMEC life span. Experiments with catalytically inactive cdc25A mutant show that phosphatase activity of cdc25A is essential for the life span extension. Our experiments show that telomerase is not activated by cdc25A expression in normal human cells, suggesting that cdc25 most likely affect M1, but not M2 checkpoints. Retroviral cDNA libraries (sense and anti-sense) were introduced into HMEC together with cdc25 to assay for the potential "enhancement" of cdc25 ability to expand the cellular life span. Among genes affected by the appropriate antisense constructs are p53, p16/ARF and seladin-1.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer, Cell Cycle	20
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction

A variety of genetic abnormalities contribute to the transformation of normal cells into aggressively growing cancer cells. Among them, loss of the normal mechanisms of proliferation control is the common theme of most human malignancies.

The cell cycle in mammalian cells is driven by the family of evolutionary conserved cyclin-dependent kinases or CDKs (CDK1-9; Morgan, 1995). As cells progress in the cell cycle, CDKs are activated and promote further passage from one cell cycle phase to another by phosphorylation of critical substrates (reviewed in Draetta, 1990; Morgan, 1995). CDKs are inactive unless associated with the cyclin subunit. So far, eight different types of cyclins have been identified in human and other higher eukaryotes (cyclins A-H; reviewed in Hunter and Pines, 1994; Morgan, 1995; Sherr, 1996). Each CDK has a preferential cyclin subunit as a partner; however, some cyclins have the ability to associate with more than one CDK, creating a large number of cyclin/kinase complexes (Morgan, 1995; Sherr, 1996). Different cyclin-CDK complexes are activated at distinct points in the cell cycle (reviewed in Sherr, 1993,1996; Sherr and Roberts, 1995; Weinberg, 1995). In addition, mammalian CDKs are negatively regulated by the diverse family of cyclin-dependent kinase inhibitors or CKIs: p16ink4/MTS1, p15ink4b/MTS2, p21cip1/WAF1/SDI1(a transcriptional target of p53), p27kip1, p57kip2 and others (reviewed in Sherr and Roberts, 1995; Harper and Elledge, 1996). These molecules specifically target CDKs important for G1-S transition: CDK2, CDK4 and CDK6. Finally, CDKs are regulated by positive (CAK, or CDK activating kinase) or negative (wee1/mik1/myt1) phosphorylation. Phosphorylation by the wee1 family of kinases is reversed by the action of cdc25 phosphatases which have a unique specificity toward CDKs (reviewed in Millar and Russel, 1992; Draetta and Eckstein, 1997). The wee1/cdc25 checkpoint was originally described in fission yeast (Russell and Nurse, 1986) and later shown to represent a universal element of the cell cycle control (Russel, Moreno and Reed, 1989; Sadhu et al., 1990; Galaktionov and Beach, 1991). All CDKs (with the exception of cdk7) contain a conserved tyrosine and in the majority of CDKs, a neighboring threonine residue, targeted by the wee1 family of kinases (Gu et al., 1992; Parker et al., 1992; Mueller et al., 1995; Terada et al., 1995; Iavarone and Massague, 1997). Phosphorylation of a kinase on either or both of these residues renders it completely inactive. In human cells, there are three known cdk-activating phosphatases, cdc25A, B and C (Sadhu et al., 1990; Galaktionov and Beach, 1991).

Transformation of the normal human cell into an aggressively growing tumor cell typically requires a series of loss of function mutations in tumor supressor genes and gain of function mutations in oncogenes. Gain of function by gene amplification has been described for c-myc and cyclin D1 in several human tumors, including breast cancer. Cyclin D1 amplification is detected in 13% of breast cancers but more than 50% appear to overexpress the protein (Sherr, 1996). Targeted overexpression of cyclin D1 in mammary epithelial cells leads to hyperproliferation and eventual tumor formation (Wang et al., 1994).

We have shown that cdc25A and cdc25B phosphatases are overexpressed in a significant portion of primary breast cancer (Galaktionov et al., 1995; Galaktionov, Chen and Beach, 1996). This overexpression might be explained in part by the action of c-myc, which is amplified in 20-30% of breast cancers, and functions in part as a transcription factor for cdc25A (Galaktionov, Chen and Beach, ibid.). Overexpression of cdc25A and cdc25B is now described in other types of human cancer as well (Gasparotto et al., 1997). Ectopic expression of cdc25A in TGFß-sensitive breast cancer cell lines renders them insensitive to the inhibitory action of this cytokine (Iavarone and Massague, 1997). As a result, cells constitutively overexpressing cdc25 phosphatases might be partially or completely insensitive to the action of TGFß, especially when the p15ink4b/mts2 (CDK4/CDK6 inhibitor, activated by TGFb) locus is inactivated by deletion. Inability of cells overexpressing cdc25 to arrest in response to negative regulatory stimuli might contribute to abnormal cell proliferation and eventually tumor formation.

The broad aim of this proposal is to investigate how perturbation of the CDK tyrosine phosphorylation checkpoint might contribute to the oncogenic transformation process.

Body

Cdc25A and cell immortalization.

As proposed in Task1, we prepared recombinant retroviruses expressing each of the three human cdc25 genes: cdc25A, cdc25B and cdc25C (Sadhu *et al.*, 1990; Galaktionov *et al.*, 1991). We used pBABE-based retroviruses since these are stable retroviral vectors and we have had previous experience working with them (Galaktionov *et al.*, 1996). To show whether the phosphatase function of cdc25 is required, we also prepared a catalytically inactive cdc25A by substituting Ser for Cys in the catalytic center of cdc25A. The ensuing titers of these viruses exceed 10^5 per ml after transient transfection into BOSC or BING cells obtained from ATCC. Mammary epithelial cells (HMEC) were obtained at passage 12 from D. Beach, Cold Spring Harbor Laboratory. Using freshly prepared retroviral stocks we were able to achieve infection rates between 10 and 40 percent routinely. This enabled us to use large infected populations without relying on single cell clones in our analysis of the potential role of cdc25 phosphatases as immortalizing agents. Infected HMECs were adjusted to the same cell count after drug selection, and growth was continued with periodic cell culture split. HMECs have a finite life span and usually exhaust their proliferation ability at about passage 20. We infected HMECs at passage 14 and counted ensuing population doublings (PDL). Control HMECs underwent only 4-5 PDLs from the point of infection before they arrested with the typical senescent cell morphology. In contrast, cells expressing cdc25A and cdc25B phosphatases continued beyond this point for 6-8 additional population doublings to total of 12-13 (Figure 1).

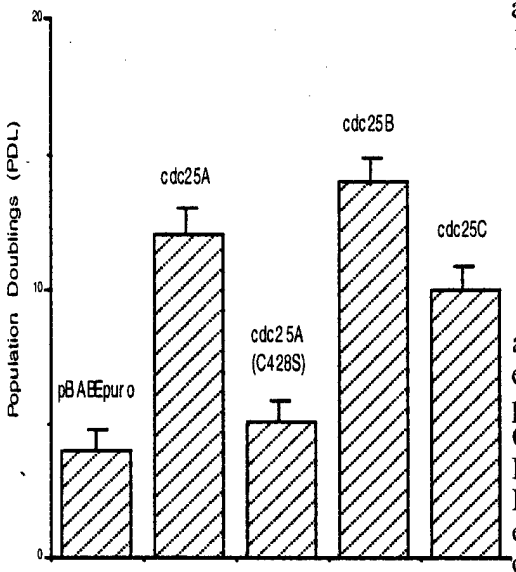


Figure 1. Life span extension by ectopic expression of cdc25 proteins. Cdc25 A, cdc25B and cdc25C, inactive cdc25A(C428S) proteins were expressed in normal human mammary epithelial cells infected with recombinant retroviruses.

We have performed these experiments three times and we are confident that cdc25 phosphatases did in fact extend the HMEC life span. The effect was most prominent with cdc25A and cdc25B phosphatases. Cdc25C phosphatase had less effect on HMEC life span. Experiments with catalytically inactive cdc25A(C482S, Figure 1) show that phosphatase activity of cdc25A is essential for the life span extension. Expression of catalytically inactive cdc25A does not cause any cell cycle arrest in HMEC, as they progress similarly to cells infected with the control retrovirus (pBABEpuro, Figure 1).

We had shown previously that a combination of oncogenic ras and cdc25A causes oncogenic transformation in rodent cells (Galaktionov *et al.*, 1995). These observations together with the experiments described here prompted us to investigate whether oncogenic rasV12 will affect the ability of cdc25A to cause life span extension in human HMEC cells. At first, we extended experiments of Serrano *et al.*, 1997, who observed that oncogenic ras causes a paradoxical reaction in normal fibroblasts, resulting in a cell cycle arrest with some evidence of the premature cell senescence (Serrano *et al., ibid.*). The cell cycle arrest caused by the ectopic expression of rasV12 requires the wild type p53 protein and is mediated at least in part by the p19ARF, encoded by the alternative reading frame of the INK4a locus (reviewed in Sherr, 1998). HMEC displays a similar phenotype as they enter the cell cycle arrest a few days after infection with pBABEpuro retrovirus expressing rasV12. The senescence phenotype was confirmed by the cell morphology (flat enlarged cells with no cell division in more than 2 weeks) and staining for the senescence-specific

b-galactosidase activity (Dimri *et al.*, 1995). Interestingly, co-expression of cdc25A or cdc25B together with rasV12 rescued HMEC cells from premature senescence caused by rasV12 (Figure 2).

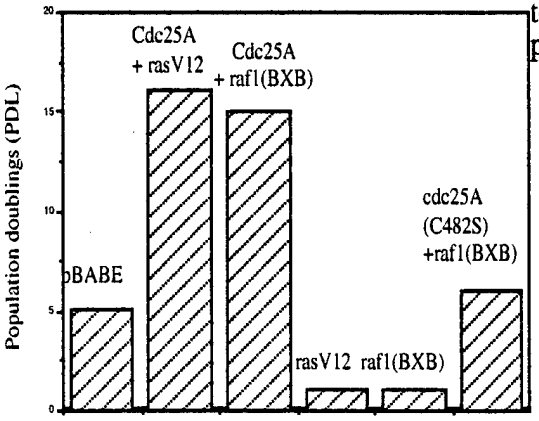
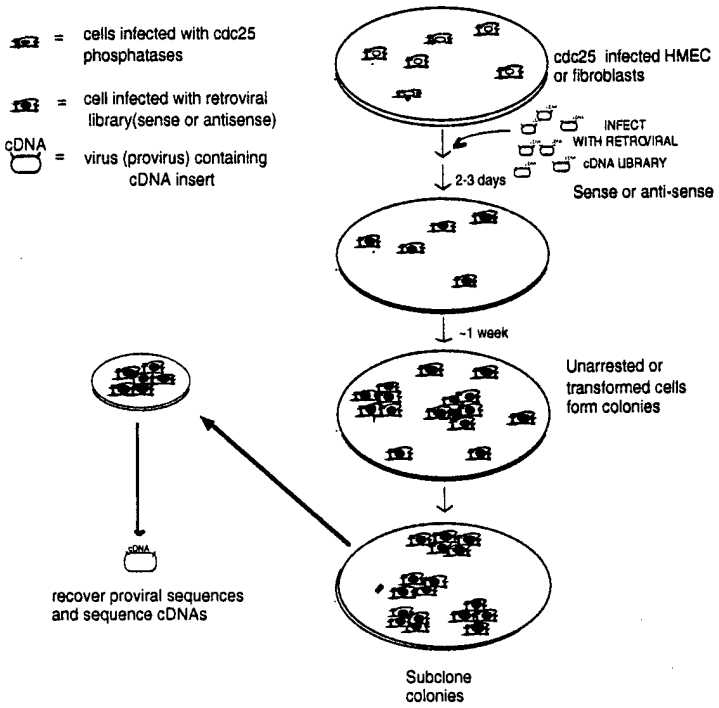


Figure 2. Life span extension by ectopic expression of cdc25A, in cooperation with rasV12 and raf1(BXB) proteins. Cdc25 A, phosphatase-"dead" cdc25A (C482S), rasV12, and raf1(BXB) proteins were expressed in normal human mammary epithelial cells infected with recombinant retroviruses. Note that rasV12 and raf1(BXB) cause a premature onset of senescence.

Because the raf/MEK/MAP kinase signaling cascade is a key effector of signaling from ras proteins, we examined the ability of raf1 kinase to affect HMEC cells. We observed that constitutively active raf1 kinase (BXB) was able to elicit cell cycle arrest and senescence (Figure 2). We wished to test whether cdc25A can reverse this arrest by co-expressing constitutively active raf1 and cdc25A. We observed that cell senescence caused by raf1 in HMEC cells was reversed by the action of cdc25A protein (Figure 2). Cells expressing both cdc25A and raf1 progress further in their life span than cdc25A alone. Interestingly, our experiments also shown that the cdc25A reversal of the cell cycle arrest caused by raf1 does not require the phosphatase activity of cdc25A, but the subsequent bypass of the normal life span does (Figure 2).

Activation of telomerase activity is often associated with the so-called M2 checkpoint, positively correlating with cell immortalization. A product of the oncogenic papilloma virus, E6, has been shown to activate telomerase activity but does not immortalize normal human mammary epithelial cells (Klingelhutz *et al.*, 1996). **As proposed in Task1**,we investigated whether this extension of the HMEC life span by cdc25 is associated with TGFβ resistance and activation of telomerase activity. To measure telomerase activity we used telomeric repeat amplification protocol (TRAP; Kim and Wu, 1997). As little as 1% telomerase-positive cells were detected by this method in a population of HMECs infected with retrovirus expressing the E6 gene product (Klingelhutz *et al., ibid.*). It has been shown recently that telomerase activity was not essential for establishment of immortal cell lines and growth of tumors in mice (Blasco *et al.*, 1997), therefore we do not necessarily expect that presence or absence of telomerase activity will represent a conclusion for our experiments, but rather another measurable parameter in addition to PDLs. The recent literature (Bodnar *et al.*, 1998), however, suggests that, in some types of human cells, ectopic expression of the catalytic telomerase subunit caused significant extension of the life span. It is possible that the contradiction between data obtained in mouse and human cells is due to species specificity and that telomerase activation is required for immortalization of the human cells. Our experiments shows that telomerase is not activated by cdc25a expression in normal human cells.

As proposed in Task1, we prepared sense and antisense cDNA libraries from polyA+ RNA isolated from growing HMEC cells. Libraries were prepared in a modified pBAbe an pWZL retroviral vectors. We began to introduce these libraries into HMEC cells that were previously infected with cdc25 expressing vectors. The overall scheme of our expreriment is shown below.



To date we isolated approximately 15 independent clones of HMEC cells where we observed the 'enhancement' of cdc25-caused expansion of life span using retroviral cDNA libraries. We are currently in the process of further characterizing these clones and attempting to identify the target genes that were either affected by antisense CDNA expression (9 clones) or express sense cDNAs (6 clones). None of sense cDNAs were able to recapitulate the observed phenomenon upon reintroduction into HMEC cells together with cdc25A. Three out of 9 antisense clones correspond to human p53 and one correspond to p16/ARF, establishing that disruption in p53 pathway cooperates with the function of cdc25 in extending the life span of HMEC. The rest of antisense clones surprisingly corresponded to recently discovered seladin-1 cDNA (5 out of 9 clones). Seladin-1 was initially uncovered in Alzheimer patients as a potential mediator of anti-oxidative response in neurons (Greeve et al., 2000). The precise mechanism of how seladin-1 interacts with cdc25 is currently under investigation. We are planning to test whether target genes require cdc25 for their function to further extend HMEC life span or they could function independently.

2. Role of wee1 kinases in establishment of the cellular senescence program
As proposed in task2, we performed low stringency PCR using degenerate oligonucleotides in order to clone putative wee1 homolog(s) with specificity toward cdk4 and cdk6 kinases. As a result, we obtain one sequence with significant sequence homology with known wee1 gene family members. However, this sequence seems to be expressed at very low levels in a majority of human tissues and cell lines. We attempted its cloning from several cDNA libraries but low representation of this clone so far prevented us from isolating a full length clone. Finally, an extensive bioinformatics analysis of recently completed human genome sequence did not reveal the additional wee1 candidate(s). It is possible that the described above cDNA fragment that had some homology to wee1 corresponded to a wee1 pseudogene. **As proposed in task2,** we performed a two-hybrid screen using modified cdk4 protein. We analysed cDNAs obtained in that screen by sequencing. So far, we have not identified a putative wee1 family member. **As proposed in task2,** we also started an attempt of biochemical purification of wee1 kinase, specific for cdk4. To that extent we have prepared a cellular extract from Hela cells and are currently characterising fractions obtained by anion exchange chromatography on FPLC trying first to identify the appropriate activity. The result of our biochemical experiments was that all fractions with kinase activity directed toward cdk2 had also activity directed toward cdk4 in vitro. We did not observe

any fractions that had activity specifically directed toward cdk4. In addition, depletion of active fractions with antibodies against wee1 and myt1 eliminated all kinase activity against cdk2 and cdk4, suggesting that human cells does not have an additional wee1 homolog. We conclude that it is likely that the task of cdk4 and cdk6 phosphorylation is accomplished by either wee1 or myt1 or both of these kinases.

KEY RESEARCH ACCOMPLISHMENTS

-cdc25 extend the life span of normal breast epithelial cells (HMEC)
-cdc25 cooperate with ras in further extending the lifespan of HMEC
-we prepared sense and antisense cDNA libraries from HMEC and introduced them into HMEC.
-Fifteen independent immortal clones were isolated using these libraries.
-among antisense cDNAs cooperating with cdc25 in extending HMEC life span we identified those corresponding to p53, p16/ARF and seladin-1.

REPORTABLE OUTCOMES

Manuscript:
Wu, L., Goodwin, E., Naeger, L., Vigo, E., **Galaktionov, K.**, Helin, K. And DiMaio, D. E2f-Rb complexes assemble and inhibit cdc25A transcription in cervical carcinoma cells following repression of human papillomavirus oncogene expression, MCB, 20: 7059-7067.
Abstract presented at Cell cycle and Cancer ISREC symposium, 1999, Lausanne, Switzerland
Abstract presented at Cell cycle meeting. Cold Spring Harbor, NY, 2000.
Abstract presented at DOD "The era of hope" conference in Atlanta, GA, 2000.
R01 grant "The cdc25 checkpoint in G1 and S phase" applied for based on findings supported by this award.

Personell receiving support from this award includes: Linna Zhang (research assistant), Ashok Kumar (postdoctoral fellow) and Irene Miloslavskaya (research assistant).

CONCLUSIONS

1. Cdc25 phosphatases expand the life span of normal mammary epithelial cells (HMEC).
2. Cdc25 phoshatases did not cause complete immortalization of HMEC
3. Phosphatase activity of cdc25A is required for the observed phenotype.
4. Cdc25 did not cause activation of telomerase in HMEC.
5. Cdc25A cooperated with RasVal12 to further expand their lifespan and reverse "senescent" phenotype caused by RasV12 in HMEC.
6. Experiments with retroviral libraries aimed at elucidating role of additional genes that might function as "enhancers" of the observed phenotypes uncovered p53, p16/ARF and seladin-1 as genes capable of cooperating with cdc25 in extending the HMEC life span. This uncovers the potential role of seladin-1 in growth control regulation.
7. The two hybrid screen with "modified" cdk4 did not yield a novel wee1 gene to date. Bioinformatics analysis of human genome did not reveal a novel wee1 homolog.

REFERENCES

Alcorta, D.A., Xiong, Y., Phelps, D., Hannon, G., Beach, D. and Barrett, J.C. (1996) Involvement of the cyclin-dependent kinase inhibitor p16 (INK4a) in replicative senescence of normal human fibroblasts. Proc. Natl. Acad. Sci.U S A 93 (24): 13742-13747.

Blasco, M.A., Lee, H.W., Hande, M.P., Samper, E., Lansdorp, P.M., DePinho, R.A. and Greider, C.W. (1997). Telomere shortening and tumor formation by mouse cells lacking

telomerase RNA. Cell 91: 25-34.
Bodnar, A.G., Ouelette, M., Frolkis, M., Holt, S.E., Chiu, C.-P., Morin, G.B., Harley, C.B., Shay, J.W., Lichtsteiner, S.B., Wright, W.E. (1998). Extension of life-span by introduction of telomerase into normal human cells. Science 278: 349-355.
Booher, R.N., Holman, P.S. and Fattaey, A. (1997). Human Myt1 is a cell cycle- regulated kinase that inhibits Cdc2 but not Cdk2 activity. J Biol Chem 272: 22300-22306.
Dimri, G.P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E.E., Linskens, M., Rubeli, I., Pereira-Smith, O., Peacocke, M., and Campisi, J. (1995). A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc Natl Acad Sci USA 92: 9363-9367.
Dowell, S.J., Romanowski, P. and Diffley, J.F. (1994). Interaction of Dbf4, the Cdc7 protein kinase regulatory subunit, with yeast replication origins in vivo. Science 265: 1243-1246.
Draetta, G. (1990). Cell cycle control in eukaryotes; molecular mechanisms of cdc2 activation. Trends. Biol.Sci. 15: 378-383.
Draetta, G. and Eckstein, J. (1997). Cdc25 protein phosphatases in cell proliferation. Biochim. Biophys. Acta 1332: M53-M63.
el-Deiry, W.S., Tokino, T., Velculescu, V.E., Levy, D.B., Parsons, R., Trent, J.M., Lin, D., Mercer, W.E., Kinzler, K.W. and Vogelstein, B. (1993). WAF1, a potential mediator of p53 tumor suppression. Cell 75: 817-825.
Fisher, R.P, and Morgan, D.O. (1994). A novel cyclin associates with MO15/CDK7 to form the CDK-activating kinase. Cell 78:713-724.
Furnari, B., Rhind, N. and Russell, P. (1997). Cdc25 mitotic inducer targeted by chk1 DNA damage checkpoint kinase. Science 277: 1495-1497.
Gabrielli, B.G., De Souza, C.P., Tonks, I.D., Clark, J.M., Hayward, N.K. and Ellem, K.A. (1996). Cytoplasmic accumulation of cdc25B phosphatase in mitosis triggers centrosomal microtubule nucleation in HeLa cells. J Cell Sci 109: 1081-1093.
Galaktionov, K. and Beach, D. (1991). Specific activation of cdc25 tyrosine phosphatases by B-type cyclins: evidence for multiple roles of mitotic cyclins. Cell 67:1181-1194.
Galaktionov, K., Lee, A.K., Eckstein, J., Draetta, G., Meckler, J., Loda, M. And Beach, D. (1995). CDC25 phosphatases as potential human oncogenes. Science 269: 1575-1577.
Galaktionov, K., Chen, X. and Beach, D. (1996). Cdc25 cell-cycle phosphatase as a target of c-myc. Nature 382: 511-517.
Gu, Y., Rosenblatt, J. and Morgan, D.O. (1992). Cell cycle regulation of cdk2 activity by phosphorylation of Thr160 and Tyr15. EMBO J. 11: 3995-4005.
Guan, K.L., Jenkins, C.W., Li, Y., Nichols, M.A., Wu, X., O'Keefe, C.L., Matera, A.G. and Xiong, Y. (1994). Growth suppression by p18, a p16INK4/MTS1- and p14INK4B/MTS2-related CDK6 inhibitor, correlates with wild-type pRb function. Genes Dev Dec 8:2939-2952.
Greeve, I., Hermans-Borgmeyer, I., Brellinger, C., Kasper, D., Gomez-isla, T., Behl, C., Levkau, B. And Notsch, R. (2000). The human DIMINUTO/DWARF1 homolog seladin-1 confers resistance to Alzheimer's desease-associated neurodegeneration and oxidative stress. The Journal of Neurosci. 20; 7345-7352.
Gudkov, A.V., Kazarov, A.R., Thimmapaya, R., Axenovich, S.A., Mazo, I.A. and Roninson, I.B. (1994). Cloning mammalian genes by expression selection of genetic suppressor elements: association of kinesin with drug resistance and cell immortalization. Proc Natl Acad Sci USA 91: 3744-3748.
Hannon, G.J., Demetrick, D. and Beach, D. (1993). Isolation of the Rb-related p130 through its interaction with CDK2 and cyclins. Genes Dev 7: 2378-2391.
Harper, J.W. and Elledge, S.J. (1996). Cdk inhibitors in development and cancer. Curr. Opin. Genet. Dev. 6: 56-64.
Hirai, H., Roussel, M.F., Kato, J.Y., Ashmun, R.A. and Sherr, C.J. (1995). Novel INK4 proteins, p19 and p18, are specific inhibitors of the cyclin D-dependent kinases CDK4 and CDK6. Mol. Cell Biol. 15: 2672-2681.

Hoffmann, I., Draetta, G. and Karsenti, E. (1994). Activation of the phosphatase activity of human cdc25A by a cdk2-cyclinE dependent phosphorylation at the G1/S transition. EMBO J 13: 4302-4310.

Hunter T, Pines J (1994). Cyclins and cancer. II: Cyclin D and CDK inhibitors come of age. Cell 79: 573-582.

Iavarone, A. and Massague, J. (1997). Repression of the CDK activator Cdc25A and cell-cycle arrest by cytokine TGF-beta in cells lacking the CDK inhibitor p15. Nature 387: 417-422.

James, P., Halladay, J. and Craig, E.A. (1996). Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. Genetics 144:1425-1436.

Jinno, S., Suto,K., Nagata,A., Igarashi,M., Kanaoka,Y., Nojima,H. and Okayama,H. (1994).Cdc25A is a novel phosphatase functioning early in the cell cycle. EMBO J. 13: 1549-1556.

Kaldis, P., Sutton, A., Solomon, M.J. (1996). The Cdk-activating kinase (CAK) from budding yeast. Cell 86 (4): 553-564.

Kim, N.W. and Wu, F. (1997). Advances in quantification and characterization of telomerase activity bythe telomeric repeat amplification protocol (TRAP). Nucleic Acids Res 25: 2595-2597.

Klingelhutz, A.J., Foster, S.A. and McDougall, J.K. (1996). Telomerase activation by the E6 gene product of human papillomavirus type16. Nature 380: 79-82.

Kumagai, A. and Dunphy, W.G. (1992). Regulation of the cdc25 protein during the cell cycle in Xenopus extracts. Cell 70: 139-151.

Kumagai, A. and Dunphy, W.G. (1996). Purification and molecular cloning of Plx1, a Cdc25 regulatory kinasefrom Xenopus egg extracts. Science 273: 1377-1380.

Lucibello, F.C., Truss, M., Zwicker, J., Ehlert, F., Beato, M. and Muller, R. (1995). Periodic cdc25C transcription is mediated by a novel cell cycle-regulated repressor element (CDE). EMBO J 14: 132-142.

Makela, T.P., Tassan, J.-P., Nigg, E.A., Frutiger, S., Hughes, G. and Weinberg, R.A. (1994). A cyclin associated with the cdk-activating kinase MO15. Nature 371:254-257.

Millar, J.B. and Russell, P. (1992). The cdc25 M-phase inducer: an unconventional protein phosphatase. Cell 68: 407-410.

Morgan, D.O. (1995). Principles of CDK regulation. Nature 374: 131-134.

Morgenstern, J.P. and Land, H. (1990). Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. Nucleic Acids Res. 18: 3587-3596.

Mueller, P.R., Coleman, T.R., Kumagai,A. and Dunphy, W.G. (1995). Myt1: a membrane-associated inhibitory kinase that phosphorylates Cdc2 on both threonine-14 and tyrosine-15. Science 270 (5233): 86-90.

Nagata, A., Igarashi, M., Jinno, S., Suto, K. and Okayama, H. (1991). An additional homolog of the fission yeast cdc25+ gene occurs in humans and is highly expressed in some cancer cells. New Biol. 3:959-968.

Noda, A., Ning, Y., Venable, S.F., Pereira-Smith, O.M. and Smith, J.R. (1994). Cloning of senescent cell derived inhibitors of DNA synthesis using an expression screen. Exp Cell Res 211: 90-98.

Parker, L.L., Atherton-Fessler, S. and Piwnica-Worms, H. (1992). p107wee1 is a dual-specificity kinase that phosphorylates p34cdc2 on tyrosine 15. Proc Natl Acad Sci U S A 89: 2917-2921.

Peng, C.Y., Graves, P.R., Thoma, R.S., Wu, Z., Shaw, A.S. and Piwnica-Worms, H. (1997). Mitotic and G2 checkpoint control: regulation of 14-3-3 protein binding by phosphorylation of Cdc25C on serine-216. Science 277: 1501-1505.

Polyak, K., Lee, M., Erdjument-Bromage, H., Koff, A., Roberts, J.M., Tempst, P. and Massague, J. (1994). Cloning of p27Kip1, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. Cell 78: 59-66.

Russell, P., Nurse, P. (1986). cdc25+ functions as an inducer in the mitotic control of fission yeast. Cell 45: 145-153.

Russell, P., Moreno,S. and Reed, S.I. (1989). Conservation of mitotic controls in fission and budding yeasts. Cell 57: 295-303.
Sadhu, K., Reed, S.I., Richardson, H. and Russell, P. (1990). Human homolog of fission yeast cdc25 mitotic inducer is predominantly expressed in G2. Proc. Natl. Acad. Sci. U S A 87: 5139-5143.
Saha, P., Eichbaum, Q., Silberman, E.D., Mayer, B.J. and Dutta, A. (1997). p21CIP1 and Cdc25A: competition between an inhibitor and an activatorof cyclin- dependent kinases. Mol Cell Biol 17: 4338-4345.
Sanchez, Y., Wong, C., Thoma, R.S., Richman, R., Wu, Z., Piwnica-Worms, H. and Elledge, S.J. (1997). Conservation of the Chk1 checkpoint pathway in mammals: linkage of DNA damage to Cdk regulation through Cdc25. Science 277: 1497-1501.
Serrano, M., Hannon, G.J. and Beach, D. (1993). A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. Nature 366:704-707.
Serrano, M., Lin , A.W., McCurrach, M.E., Beach, D and Lowe,S.W. (1997). Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell 88: 593-602.
Sherr, C.J. (1993). Mammalian G1 cyclins. Cell 73: 1059-1065.
Sherr, C.J. (1996). Cancer cell cycles. Science 274:1672-1677.
Sherr, C.J. (1998). Tumor surveillance via the ARF-p53 pathway. Genes Dev. 12:2984- 2991.
Sherr, C.J. and Roberts, J.M. (1995). Inhibitors of mammalian G1 cyclin-dependent kinases. Genes Dev. 9: 1149-1163.
Simmons, D.L., Neel, B.G., Stevens, R., Evett, G. and Erikson, R.L. (1992). Identification of an early growth-response gene encoding a novel putative protein kinase. Mol Cell Biol 12: 4164-4169.
Spitkovsky, D., Jansen-Durr, P., Karsenti, E. and Hoffman, I. (1996). S-phase induction by adenovirus E1A requires activation of cdc25a tyrosine phosphatase. Oncogene 12: 2549-2554.
Terada, Y., Tatsuka, M., Jinno, S., and Okayama, H. (1995). Requirement for tyrosine phosphorylation of Cdk4 in G1 arrest induced by ultraviolet irradiation. Nature 376:358-362.
Walworth, N.C. and Bernards, R. (1996). Rad-dependent response of the chk1-encoded protein kinase at the DNA damage checkpoint. Science 271: 353-356.
Watanabe, N., Broome, M. and Hunter,T. (1995). Regulation of the human WEE1Hu CDK tyrosine 15-kinase during the cell cycle. EMBO J. 14: 1878-1891.
Weinberg, R.A. (1995). The retinoblastoma protein and cell cycle control. Cell 81: 323- 330.
Wright, W.E., Pereira-Smith, O.M. and Shay, J.W. (1989). Reversible cellular senescence: implications for immortalization of normal human diploid fibroblasts. Mol Cell Biol 9: 3088-3092.

Appendices

A reprint of the aforementioned manuscript by Wu *et al.* is attached.

MOLECULAR AND CELLULAR BIOLOGY, Oct. 2000, p. 7059–7067 Vol. 20, No. 19
0270-7306/00/$04.00+0

E2F-Rb Complexes Assemble and Inhibit cdc25A Transcription in Cervical Carcinoma Cells following Repression of Human Papillomavirus Oncogene Expression

LINGLING WU,[1] EDWARD C. GOODWIN,[1] LISA KAY NAEGER,[1]† ELENA VIGO,[2] KONSTANTIN GALAKTIONOV,[3] KRISTIAN HELIN,[2] AND DANIEL DiMAIO[1]*

Department of Genetics, Yale University School of Medicine, New Haven, Connecticut 06510[1]; Department of Experimental Oncology, European Institute of Oncology, 20141 Milan, Italy[2]; and Department of Molecular and Human Genetics, BCM-Ben Taub Research Center, Baylor College of Medicine, Houston, Texas 77030[3]

Received 4 April 2000/Returned for modification 15 May 2000/Accepted 5 July 2000

Expression of the bovine papillomavirus E2 protein in cervical carcinoma cells represses expression of integrated human papillomavirus (HPV) E6/E7 oncogenes, followed by repression of the cdc25A gene and other cellular genes required for cell cycle progression, resulting in dramatic growth arrest. To explore the mechanism of repression of cell cycle genes in cervical carcinoma cells following E6/E7 repression, we analyzed regulation of the cdc25A promoter, which contains two consensus E2F binding sites and a consensus E2 binding site. The wild-type E2 protein inhibited expression of a luciferase gene linked to the cdc25A promoter in HT-3 cervical carcinoma cells. Mutation of the distal E2F binding site in the cdc25A promoter abolished E2-induced repression, whereas mutation of the proximal E2F site or the E2 site had no effect. None of these mutations affected the activity of the promoter in the absence of E2 expression. Expression of the E2 protein also led to posttranscriptional increase in the level of E2F4, p105^{Rb}, and p130 and induced the formation of nuclear E2F4-p130 and E2F4-p105^{Rb} complexes. This resulted in marked rearrangement of the protein complexes that formed at the distal E2F site in the cdc25A promoter, including the replacement of free E2F complexes with E2F4-p105^{Rb} complexes. These experiments indicated that repression of E2F-responsive promoters following HPV E6/E7 repression was mediated by activation of the Rb tumor suppressor pathway and the assembly of repressing E2F4-Rb DNA binding complexes. Importantly, these experiments revealed that HPV-induced alterations in E2F transcription complexes that occur during cervical carcinogenesis are reversed by repression of HPV E6/E7 expression.

Cells have evolved complex regulatory mechanisms to ensure orderly progression through the cell cycle. One of the major regulatory systems entails the interactions between members of the retinoblastoma susceptibility (Rb) protein family and E2F transcription factors. p105^{Rb} and other members of the Rb family, p107 and p130, form complexes with various members of the E2F family and regulate their activity (15, 43). E2F transcription factors exist as stable heterodimers with DP subunits. During the G_1 and G_0 phases of the cell cycle, complexes consisting of E2F-DP heterodimers and hypophosphorylated Rb proteins actively repress promoters that contain E2F binding sites (21, 25, 27, 33, 35, 40, 42, 58, 61). Many of the genes repressed in this fashion encode proteins that are required for entry into and transit through S phase, and E2F4-p105^{Rb} and E2F4-p130 complexes are particularly active in transcriptional repression (9, 39, 53, 54, 57). In addition, complex formation with Rb family members protects E2F proteins from degradation by the ubiquitin-proteosome pathway and promotes the localization of E2F4 to the nucleus (22, 26, 37, 38). In contrast, phosphorylation of Rb family members by cyclin-dependent kinases during cell cycle progression disrupts Rb-containing E2F complexes and releases free E2F-DP heterodimers that may then act as transcriptional activators at promoters containing E2F binding sites (15, 43). The importance of E2F-Rb complexes in regulating cell growth is underscored by the finding that diverse DNA tumor viruses encode proteins that disrupt these complexes, leading to uncontrolled cell growth (44).

The genes encoding p53 and p105^{Rb} are frequently mutant in a variety of human cancers. In contrast, cervical carcinomas and carcinoma-derived cell lines often contain wild-type tumor suppressor genes (7, 46). These cancers almost invariably harbor high-risk human papillomavirus (HPV) genomes and express the viral oncogenes E6 and E7 (56). The high-risk HPV E6 and E7 proteins bind to p53 and p105^{Rb} (and other Rb members), respectively, and neutralize their growth-inhibitory function. The E6 protein targets p53 for ubiquitin-mediated proteolysis (47). Similarly, the E7 protein targets Rb family members for ubiquitin-mediated proteolysis, resulting in decreased Rb levels in cells expressing the viral protein (1, 3, 34). In addition, the E7 protein sequesters Rb proteins so that free E2F is released (4). Cells expressing high-risk E6 and E7 proteins display impaired checkpoint control following DNA damage and exhibit elevated rates of mutagenesis (10, 11, 23, 24, 50, 59). Thus, even though cervical carcinoma cells often maintain wild-type p53 and p105^{Rb} genes, tumor suppressor activity is largely eliminated, implying that HPV-infected cervical epithelial cells are subjected to continuing genetic insults which may ultimately result in irreversible loss of growth control.

In contrast to the HPV E6 and E7 genes, the HPV E2 gene is frequently disrupted in cervical carcinomas (56), presumably reflecting the ability of the papillomavirus E2 proteins to bind directly to the HPV early promoter and repress transcription of the E6 and E7 genes (2). Ectopic expression of HPV or

* Corresponding author. Mailing address: Department of Genetics, Yale University School of Medicine, 333 Cedar St., New Haven, CT 06510. Phone: (203) 785-2684. Fax: (203) 785-7023. E-mail: daniel.dimaio@yale.edu.

† Present address: Gilead Sciences, Foster City, CA 94404.

bovine papillomavirus (BPV) E2 proteins in cervical carcinoma cell lines such as HeLa or HT-3 cells, which contain HPV type 18 (HPV18) or HPV30 DNA, respectively, results in the specific and rapid repression of the endogenous HPV E6 and E7 genes and in significant growth inhibition, with the inhibited cells accumulating with G_0/G_1 DNA content (12, 13, 29, 30, 41). Several lines of indirect evidence indicate that E2-induced growth inhibition is mediated by repression of E6/E7 expression (20, 41), and it has recently been shown that constitutive expression of the HPV16 E6/E7 proteins can block the growth-inhibitory effect of the E2 protein (17; Goodwin and DiMaio, submitted). Expression of the E2 protein and repression of HPV E6 and E7 expression results in the subsequent accumulation of hypophosphorylated $p105^{Rb}$ and repression of the E2F1, cyclin A, and cdc25A genes (29, 41; Goodwin and DiMaio, submitted). cdc25A encodes a protein tyrosine phosphatase that can remove inhibitory phosphates from cyclin-dependent kinases and is required for cell cycle progression in cervical carcinoma cells (reviewed in reference 14). Taken together, these results suggest that growth-regulatory pathways in the cervical cancer cells are largely intact but dormant until they are mobilized by the E2 protein. Thus, the E2 protein can be used as a reagent to repress E6/E7 expression, reactivate tumor suppressor pathways, and explore the consequences on cell physiology. Unlike experiments involving introduction of foreign cellular genes, this approach induces expression of endogenous tumor suppressor proteins, an experimental design less prone to artifacts due to protein overexpression or interactions between heterologous proteins.

Here, we analyzed the regulation of the human cdc25A promoter in cervical carcinoma cells in response to the E2 protein and repression of E6/E7 expression. We show that the E2 protein represses cdc25A transcription by inducing the assembly of functional E2F-Rb transcriptional repressor complexes that bind to the cdc25A promoter. Thus, the alterations in the composition and function of E2F-Rb transcription complexes that occur during cervical carcinogenesis are reversible.

MATERIALS AND METHODS

DNAs. The *Xho*I-to-*Xba*I fragment containing the human cdc25A promoter was isolated from genomic clone pBSK-cdc25A 12E (18) and inserted into *Xho*I-plus-*Nhe*I-digested pGL3 basic (Promega) to generate pGL3b-cdc25A-XX. E2 site mutations were constructed in pGL3b-cdc25A-BX, which contains the *Bam*HI-to-*Xho*I fragment of the human cdc25A promoter, by using a QuikChange mutagenesis kit (Stratagene). The luciferase reporter containing the wild-type *Sac*I promoter fragment and fragments containing mutations in the E2F sites were described previously (55).

Cells and virus stocks. HT-3 cells were maintained in McCoy's 5A medium containing 15% fetal bovine serum. BPV1-simian virus 40 (SV40) recombinant virus stocks were prepared and titered as described previously (41). To generate HT-3 cells containing a stably integrated reporter plasmid, 5×10^5 cells were plated in a 60-mm-diameter tissue culture dish. When the cells were 80% confluent, 12 μl of Lipofectamine was mixed with 5 μg of pGL3b-cdc25A-XX and 0.5 μg of pBabe-puro in 3 ml of Opti-MEM medium, and the transfection mixture was incubated with the cells for 6 h (20). At 72 h after transfection, the cells were passaged in 1:10 into medium containing 1 μg of puromycin per ml. Individual puromycin-resistant colonies were isolated, expanded into cell lines, and tested for luciferase expression in the absence of E2 expression. The cell line displaying the highest basal level of luciferase activity was used for further experiments.

Infection, transfection, and luciferase assays. HT-3 cells were plated at a density of 10^5 cells/well in a 24-well tissue culture plate and grown overnight. The cells were then infected with recombinant virus in 200 μl of Dulbecco's modified Eagle's medium containing 2% fetal bovine serum for 5 h with tilting at 37°C. The cells were washed twice with phosphate-buffered saline (PBS) and transfected with 1 μg of firefly luciferase reporter plasmids by using 2.5 μl of Lipofectamine as described above. Cells were lysed in 200 μl of cell culture lysis reagent 48 h after infection, and luciferase activity was measured by using the Promega luciferase assay system. Each experiment was performed in duplicate or triplicate, and the results shown in each figure are the average for a representative experiment, with the error bars showing standard deviation.

Northern blot analysis. Total RNA was prepared from HT-3 cells with Trizol (Life Technologies) 48 h after mock infection or infection at a multiplicity infection (MOI) of 20, subjected to formaldehyde-agarose gel electrophoresis, and transferred to Nytran supercharge (Scheicher and Schuell). E2F cDNAs labeled with [α-^{32}P]dATP by random priming were used to probe replicate filters.

Preparation of cell extracts. (i) Nuclear extracts. HT-3 cells were plated at 2.2×10^6 cells per 10-cm-diameter culture dish, grown overnight, and then mock infected or infected with virus at an MOI of 20. At 24, 48, or 60 h after infection, cells were washed twice with PBS and then lysed in 800 μl of buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol [DTT], 0.5 mM phenylmethylsulfonyl fluoride) for 15 min on ice. After addition of 50 μl of 10% NP-40 (Sigma), vigorous vortex mixing, and brief microcentrifugation, the nuclear pellet was resuspended in 100 μl of buffer C (20 mM HEPES [pH 7.9], 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride and incubated on ice for 20 min. After centrifugation in a microcentrifuge at maximum speed for 10 min, the supernatant was used immediately or stored at −70°C.

(ii) Whole-cell extracts. At 48 h after infection, cells were washed twice with cold PBS and scraped in 10 ml of cold PBS. The cells were centrifuged and suspended in lysis buffer (20 mM HEPES [pH 7.6], 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol) supplemented with proteinase inhibitors. Extracts were stored at −70°C.

Proteosome inhibitor studies. HT-3 cells were plated at a density of 2.2×10^6 cells/100-mm-diameter tissue culture dish. After overnight growth, the cells were incubated for 12 h with LLnL (50 μg/ml; Sigma) or MG132 (20 μg/ml; Calbiochem) in McCoy's 5A medium containing 15% fetal bovine serum. The cells were then washed twice with cold PBS, and whole-cell extracts were prepared by lysis in 0.1% NP-40 lysis buffer (250 mM NaCl, 0.1% NP-40, 50 mM Tris-Cl, 5 mM EDTA) containing protease inhibitors. Extracted protein (2 mg) was immunoprecipitated and immunoblotted with the appropriate monoclonal antibodies. As controls, cells were also harvested without inhibitor treatment 48 h after infection with the E2 virus or after mock infection.

Western blot and immunoprecipitation analyses. Western blots were performed with whole-cell extracts, unless specified differently in a figure legend. Extracted protein (25 μg) was electrophoresed in a sodium dodecyl sulfate–11% polyacrylamide gel for E2F1 and E2F4 blots. The separated proteins were transferred to nitrocellulose membrane and blotted with antibodies (Upstate Biotechnology, catalogue no. 05-379 [E2F1] or 05-312 [E2F4]) in PBS containing 3% nonfat milk.

Immunoprecipitation was performed by adding 2 μg of E2F4 antibody (sc-866; Santa Cruz Biotechnology, Inc.) or nonimmune rabbit serum to 200 μg of total protein. After incubation at 4°C overnight, 50 μl of protein A/G PLUS-Agarose (Santa Cruz) was added, incubation was continued for another 2 h, and then the mixture was washed four times with lysis buffer. The antibodies used to probe Western blots of Rb family members were 140010 (Pharmingen) for $p105^{Rb}$, sc-317 (Santa Cruz) for p130, and sc-318 (Santa Cruz) for p107.

Mobility shift assays. Mobility shift assays were performed as described by Hurford et al. (29) with a double-stranded oligonucleotide probe end labeled with polynucleotide kinase and [γ-^{32}P]ATP (top strand, 5′-GTGGATTCCGTT TGGCGCCAACTAGGAAAG-3′; nucleotides [nt] −72 to −43 of the human cdc25A promoter containing E2F binding site 1). Fresh nuclear extracts containing 20 μg of extracted protein were incubated with 0.5 to 1 ng of end-labeled probe for 15 min at room temperature in 15 μl of reaction buffer containing 25 μg of sonicated salmon sperm DNA per ml, 20 mM HEPES (pH 7.5), 4% Ficoll 400-DL, 2.5 mM $MgCl_2$, 40 mM KCl, 0.1 mM EGTA, 2 mM spermine, 0.5 mM DTT, and 0.5 μg of acetylated bovine serum albumin (Gibco-BRL) per μl. The samples were loaded onto a 4% native polyacrylamide gel prerun at 100 V for 30 min in 0.25× Tris-borate-EDTA buffer at 4°C and then electrophoresed at 180 V for 3 h. The gel was fixed with 10% acetic acid for 10 min, dried, and exposed. Unlabeled double-stranded oligonucleotides containing the wild-type or mutant E2F site 1 or the wild-type E2F site 2 (top strand, 5′-GCCGCTATTACCGCG AAAGGCCGGCCTGGC-3′) were used as competitors. Supershifts were performed by addition of the appropriate antibodies (0.8 μg of anti-human E2F1 Powerclonal [Upstate Biotechnology] for E2F1 supershift, sc-999 [Santa Cruz] for E2F5, and 140010 [Pharmingen] for $p105^{Rb}$; 4 μl of WuF-10 monoclonal antibody [obtained from F. Dick] for E2F4) to the mixture of protein and probe and incubation for another 20 min prior to electrophoresis.

RESULTS

Analysis of cdc25A promoter activity by using an integrated reporter construct. We previously showed that acute expression of the BPV E2 protein caused a dramatic reduction in HPV E6/E7 expression in HeLa and HT-3 cervical carcinoma cells, followed by repression of endogenous cdc25A mRNA and protein (30, 41; Goodwin and DiMaio, submitted). Since HT-3 cells contain transactivation-defective p53 (41), we focused on these cells to explore regulation of the cdc25A gene

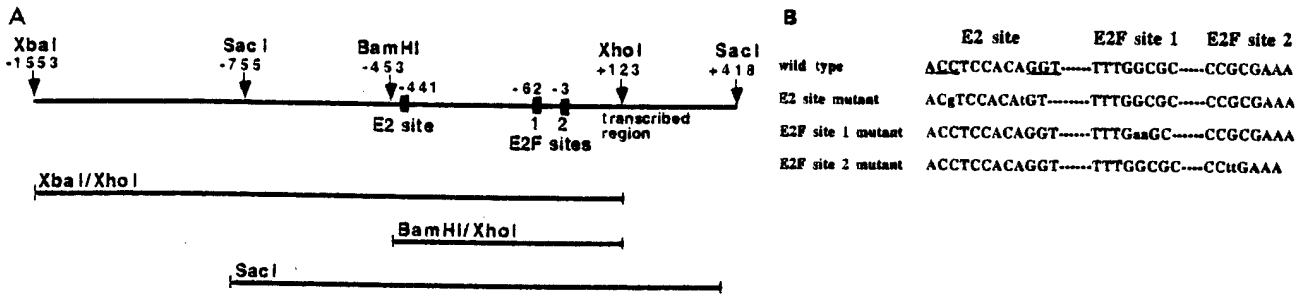


FIG. 1. (A) Map of the human cdc25A promoter. The top line shows the positions of restriction endonuclease cleavage sites and consensus binding sites for the E2 protein and E2F. Numbers indicate the number of nucleotides from the transcription start site. Lines at the bottom part show the promoter fragments used in this study. (B) Sequences of the wild-type and mutant transcription factor binding sites in the cdc25A promoter. The invariant nucleotides that constitute the consensus E2 recognition site are underlined. Mutant nucleotides are shown in lowercase.

in the absence of a p53 response. To determine whether the *cis*-acting elements responsible for cdc25A repression resided in the 5′ flanking region of the gene, we carried out experiments that utilized a reporter gene linked to the human cdc25A promoter. A 1,676-bp *Xba*I-to-*Xho*I fragment of the promoter extending from nt −1553 to +123 relative to the transcription start site was cloned upstream of the firefly luciferase gene (Fig. 1A). Initiation of cdc25A translation occurs downstream of the *Sac*I site at nt +418. This reporter construct was stably introduced into HT-3 cells by selection for a cotransfected puromycin resistance gene.

HT-3 cells containing the integrated cdc25A promoter-luciferase gene were infected at various MOIs with a BPV-SV40 recombinant virus that expresses a wild-type BPV E2 protein. At 48 h after infection, cells were harvested and extracts were assayed for luciferase activity. As shown in Fig. 2A, virus infection caused a dose-dependent reduction in luciferase activity. At high MOI, luciferase activity was reduced approximately fivefold. In contrast, infection with a virus expressing an inactive E2 truncation mutant did not repress the cdc25A promoter. These experiments provided evidence that repression of the cdc25A gene was at the transcriptional level and demonstrated that the 5′ flanking region of the cdc25A gene contained elements that responded to the E2 protein.

Identification of elements of the cdc25A promoter that mediate repression. To carry out a mutational analysis of the cdc25A promoter, cells were infected at high MOI with recombinant viruses expressing wild-type or mutant E2 proteins, incubated for 5 to 6 h, and then transfected with various luciferase-based reporter plasmids. Extracts harvested 48 h after infection were assayed for luciferase activity. Although the wild-type E2 protein did not repress the luciferase gene driven by the SV40 early promoter, it caused an approximately fivefold repression of the luciferase gene driven by the *Xba*I-*Xho*I fragment containing the wild-type cdc25A promoter (Fig. 2B). In contrast, the cdc25A promoter was not repressed by infection with viruses expressing a truncated E2 mutant or a DNA binding-defective E2 point mutant, neither of which repressed the endogenous cdc25A gene (20, 41).

The segment of the cdc25A promoter conferring E2 responsiveness contained a single consensus binding site for the E2 protein itself located at nt −441 to −430 and two consensus E2F binding sites near the transcription start site (Fig. 1A). To test whether these elements were responsible for E2-mediated repression of the cdc25A reporter, we constructed mutations at these sites, transiently transfected each mutant construct into HT-3 cells, infected the cells 6 h later with viruses expressing the E2 protein, and measured luciferase activity after an additional 42 h. The mutations tested are shown in Fig. 1B. To destroy the E2 binding site, two point mutations were introduced into the consensus sequence absolutely required for E2 DNA binding. These mutations were introduced into a 576-bp *Bam*HI-to-*Xho*I promoter fragment (nt −453 to +123). The wild-type promoter fragment was repressed approximately fourfold by the E2 protein, indicating that elements responsible for E2-induced repression resided in this fragment (Fig. 3A). The mutations in the E2 site had no significant effect on the basal level of cdc25A promoter activity, and they did not affect E2-mediated repression (Fig. 3A). Thus, an intact E2 binding site was not required for E2-mediated repression of the human cdc25A promoter.

We next tested the effect of mutations in the two E2F consensus sites in the cdc25A promoter. We designate the site at nt −3 to +5, more proximal to the transcription start site, as site 2 and the distal site at nt −62 to −55 as site 1. Mutations known to prevent E2F binding to site 1 or site 2 were introduced into a 1,173-bp *Sac*I fragment (nt −755 to +418) containing the cdc25A promoter (55). As shown in Fig. 3B, the wild-type *Sac*I promoter fragment was efficiently repressed by the wild-type E2 protein. The level of luciferase expression in the absence of the E2 protein was not affected by mutation of either E2F site, and mutation of E2F binding site 2 did not interfere with E2-mediated repression. Strikingly, however, mutations in E2F site 1 abolished E2-mediated repression of the cdc25A promoter. Similar results were obtained in four independent transfection experiments. Thus, E2-mediated repression of the cdc25A promoter did not appear to be a direct effect of the E2 protein binding to the promoter but rather required an intact E2F site located approximately 60 bp upstream from the transcription start site. Mutations at two additional elements between the two E2F sites, a CHR site and an Sp1 site, did not affect E2-mediated repression (data not shown).

Expression of E2F and Rb family members in response to the E2 protein. The simplest explanation for the results presented above is that the E2 protein induced the synthesis of a protein that repressed promoter activity and that this protein must bind to E2F site 1 to exert this repressing effect. E2F family members, when bound to Rb family members, can repress transcription from promoters containing E2F sites, suggesting that E2F-Rb complexes might be this putative repressor. We previously showed that the E2 protein caused a dramatic increase in the level of hypophosphorylated $p105^{Rb}$ and a decrease in the level of E2F1 in HeLa and HT-3 cells (29, 41). Here, we used Western blotting to assess the levels of all three Rb proteins, $p105^{Rb}$, p107, and p130, 2 days after introduction of the E2 gene. As shown in Figure 4A (right), we confirmed the induction of the full-length hypophosphorylated

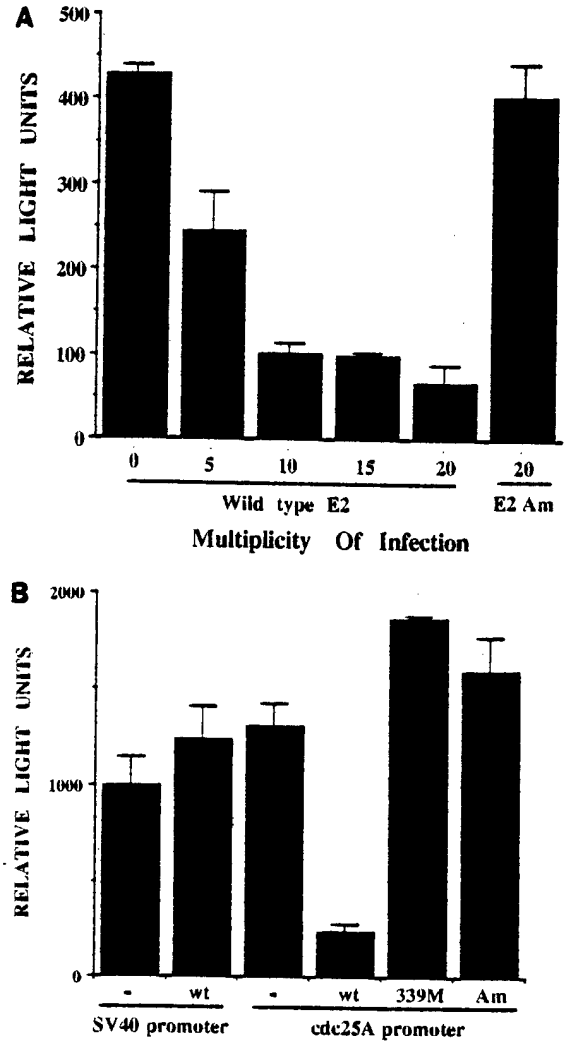


FIG. 2. E2-mediated repression of the cdc25A promoter. (A) HT-3 cells harboring a stably integrated cdc25A *Xba*I-to-*Xho*I promoter fragment linked to the luciferase gene were infected at the indicated MOI with recombinant viruses expressing the wild-type or a nonsense mutant (E2 Am) E2 protein. Luciferase activity was measured on cell extracts prepared 48 h after infection. (B) Cells were mock infected (−) or infected with viruses expressing the wild-type E2 protein (wt), the DNA binding-defective mutant K339M (339M), or the E2 nonsense mutant (Am) at an MOI of 20. After 5 to 6 h, the cells were transfected with the luciferase gene linked to the SV40 early promoter or to the wild-type *Xba*I-to-*Xho*I cdc25A promoter fragment; after an additional 2 days, cells were harvested and luciferase activity was measured.

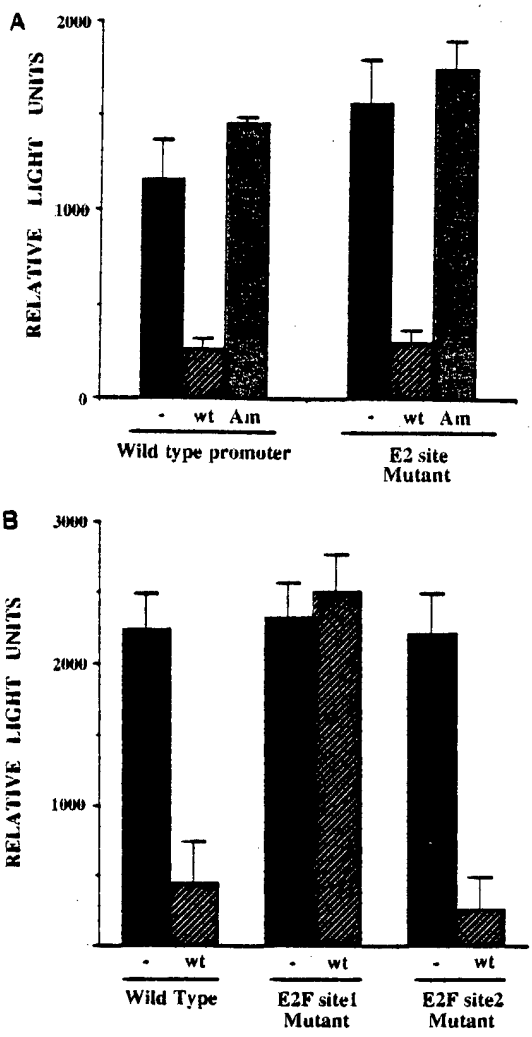


FIG. 3. Analysis of the cdc25A promoter containing mutations in transcription factor binding sites. (A) E2 binding site. Cells were mock-infected (−) or infected with a virus expressing the wild-type (wt) or nonsense mutant (Am) E2 protein, as indicated. After 5 to 6 h, the cells were transfected with the luciferase gene linked to the wild-type *Bam*HI-*Xho*I fragment of the cdc25A promoter or the fragment containing two mutations in the consensus E2 binding site, and luciferase activity was determined. (B) E2F sites. Cells were mock infected (−) or infected with a virus expressing the wild-type E2 protein (wt). After 5 to 6 h, cells were transfected with the luciferase gene linked to the wild-type *Sac*I fragment of the cdc25A promoter or fragments containing mutations in either E2F site 1 or E2F site 2, and luciferase activity was determined.

form of p105^{Rb}. (HT-3 cells also express a deleted version of p105^{Rb}, whose expression is not affected by E2 expression [41].) The level of p130 also markedly increased in response to E2 expression, whereas p107 expression was unchanged (Fig. 4A, right). Because of the well-known repressing activity of E2F4 when it is associated with Rb family members, we also assessed the expression of E2F4. There was a dramatic increase in the amount of E2F4 in HT-3 nuclear extracts (and in whole-cell extracts [see Fig. 5]) in response to expression of the E2 protein, whereas in confirmation of earlier results, E2F1 expression was inhibited by the E2 protein (Fig. 4A, right).

We used Northern blotting to explore the mechanism by which the E2 protein affected the concentration of Rb and E2F family members. As shown in Fig. 4A (left), E2 expression did not cause an increase in the levels of the mRNA encoding p105^{Rb}, p130, or E2F4. Therefore, the induction of these proteins in response to E2 expression did not reflect increased transcription or mRNA stability. In contrast, E2F1 mRNA was reduced, as reported earlier. The levels of E2F3 and E2F5 mRNA did not change in response to E2 expression (data not shown).

The results presented above demonstrated that the E2 protein caused posttranscriptional induction of p105^{Rb}, p130, and E2F4. The relatively low concentrations of endogenous E2F4 and p105^{Rb} in proliferating HT-3 cells prevented us from directly measuring changes in the half-life of these proteins in response to E2 expression. As an alternate approach, we tested whether E2F4 and p105^{Rb} levels were controlled by proteosome-mediated proteolysis in cervical carcinoma cells, as they are in other cell types (1, 3, 34). Cells were incubated with the peptide aldehyde LLnL or MG132, specific inhibitors of the

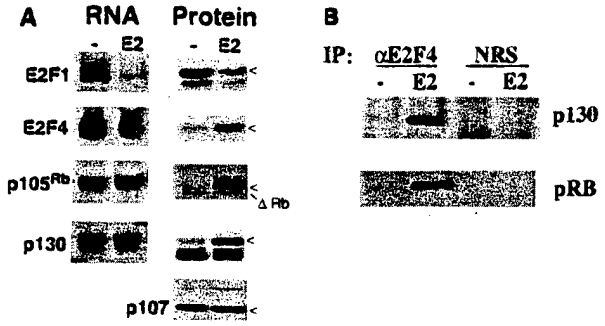


FIG. 4. (A) Expression of E2F and Rb family members. (Right) Nuclear (top two panels) and whole-cell (bottom three panels) extracts were prepared 48 h after mock infection (−) or infection with a virus expressing the wild-type E2 protein (E2). After gel electrophoresis and transfer, filters were probed with antibody recognizing p105^{Rb}, p107, p130, E2F4, or E2F1, as indicated. ΔRb is the deleted form of p105^{Rb} expressed in HT-3 cells. (Left) RNA was isolated 48 h after mock infection (−) or after infection with a virus expressing the wild-type E2 protein (E2). After gel electrophoresis and transfer, filters were hybridized to the indicated radiolabeled cDNA probes. (B) Complex formation between E2F4 and Rb family members. Nuclear extracts from E2-infected and mock-infected cells were immunoprecipitated (IP) with nonimmune rabbit serum (NRS) or anti-E2F4 (αE2F4) rabbit antiserum, electrophoresed, and transferred. The filter was then probed sequentially with antibodies recognizing p105^{Rb} and p130, as indicated.

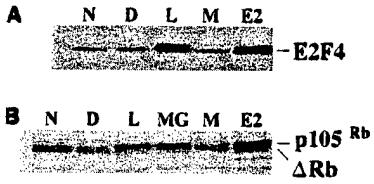


FIG. 5. Effect of proteosome inhibitors on E2F4 and p105^{Rb} levels. Cells were mock infected (M), infected with a virus expressing the wild-type E2 protein (E2), treated with LLnL (L) or MG132 (MG) in DMSO, treated with DMSO alone (D), or incubated in normal medium (N), as indicated. NP-40 whole-cell lysates were prepared, and E2F4 (A) or p105^{Rb} (B) was immunoprecipitated from equal amounts of extracted protein, electrophoresed, transferred to filters, and detected by probing the filters with specific antibodies.

26S proteosome, or dimethyl sulfoxide (DMSO) alone. In parallel, cells were mock-infected or infected with the E2 virus. We then performed immunoprecipitation and Western blotting on equal amounts of total cell protein to examine levels of p105^{Rb} and E2F4. This analysis was performed with whole-cell lysates, to eliminate relocalization to the nucleus as a cause of apparent differences in expression levels. As shown in Fig. 5A, in mock-infected cells, most of the E2F4 migrated as a single band upon gel electrophoresis. Following LLnL treatment or expression of the E2 protein, the amount of E2F4 in this band increased, and multiple additional E2F4 bands with different electrophoretic mobilities were detected. Similar results were obtained when cells were treated with MG132 (data not shown). The more complex pattern of E2F4 expression in whole cells compared to nuclear extracts (Fig. 4A, right) presumably reflects the presence of additional modified E2F4 species in nonnuclear locations. Similarly, LLnL or MG132 treatment of uninfected cells or expression of the E2 protein resulted in increased levels of full-length p105^{Rb} but not the deleted form (Fig. 5B). We conclude that E2F4 and p105^{Rb} are subject to rapid proteosome-mediated degradation in proliferating HT-3 cells. Furthermore, these results suggest that the E2 protein may directly or indirectly protect these cell cycle-regulatory proteins from this fate, contributing to the increased concentration that is observed. However, these experiments do not rule out the possibility that the E2 protein exerts additional effects on the expression of p105^{Rb} or E2F4.

Because complexes containing E2F4 and Rb family members can repress transcription, we determined whether E2 expression induced the formation of such complexes. We carried out immunoprecipitation with a rabbit polyclonal anti-E2F4 antibody and immunoblotted the resulting immunoprecipitates with antibodies that recognize p105^{Rb} or p130. As shown in Fig. 4B, the anti-E2F4 antibody immunoprecipitated little, if any, p130 or p105^{Rb} from nuclear extracts of uninfected cells. In contrast, E2 expression caused a dramatic increase in the amount of p105^{Rb} and p130 coimmunoprecipitated by the anti-E2F4 antibody. Similar results were obtained by immunoprecipitation with a monoclonal antibody that recognizes E2F4 (data not shown), and nonimmune rabbit antiserum did not immunoprecipitate the Rb family members (Fig. 4B). E2 expression did not affect the level of p107 in complex with E2F4 (data not shown). Therefore, expression of the E2 protein and repression of HPV30 E6 and E7 resulted in elevated levels of E2F4-p105^{Rb} and E2F4-p130 complexes in the nuclei of HT-3 cells.

Oligonucleotide mobility shift analysis. We carried out oligonucleotide mobility shift experiments to examine the protein complexes capable of forming on E2F site 1, which mediates E2 repression of the cdc25A promoter. A 30-bp double-stranded oligonucleotide spanning this site was end labeled and incubated with nuclear extracts of uninfected HT-3 cells and of cells infected with the virus expressing the wild-type E2 protein. After electrophoresis through a nondenaturing gel, DNA-protein complexes were visualized by autoradiography. As shown in Fig. 6A, nuclear extracts from mock-infected HT-3 cells gave rise to three prominent retarded species, labeled A, B, and C. Expression of the E2 protein caused a dramatic change in the pattern of retarded bands. Band A persisted and was reproducibly more abundant following E2 expression, band B was markedly reduced in intensity, and two new major bands (D and E) appeared that comigrated with minor bands generated by the mock extracts. Band C was generated in similar amounts by both samples. To examine the specificity of these complexes, competition studies were carried out. Bands A, B, D, and E were competed by excess unlabeled oligonucleotide containing the wild-type E2F site 1 but not by an oligonucleotide containing the same mutation that abolished repression (Fig. 6A). In addition, these bands were not observed when a mutant oligonucleotide was used as a probe (data not shown). Thus, bands A, B, D, and E represent specific E2F complexes whose abundance varies in response to E2 expression. In contrast, band C was not competed by the unlabeled wild-type oligonucleotide, indicating that it was not a specific complex. Therefore, expression of the E2 protein and repression of E6/E7 expression resulted in a dramatic alteration in the protein complexes that specifically bound E2F site 1. If infection was allowed to proceed for 60 h or if it was carried out at higher MOI, band D was by far the most abundant complex formed at E2F site 1 (Fig. 6B and data not shown).

To identify the proteins present in complexes forming at E2F site 1, we performed supershift analysis with specific antibodies (Fig. 7). Band B was supershifted by the E2F1 antibody, generating band b. This is consistent with the reduction of intensity of band B in response to the E2 protein, which represses E2F1 expression. In contrast, band A generated by

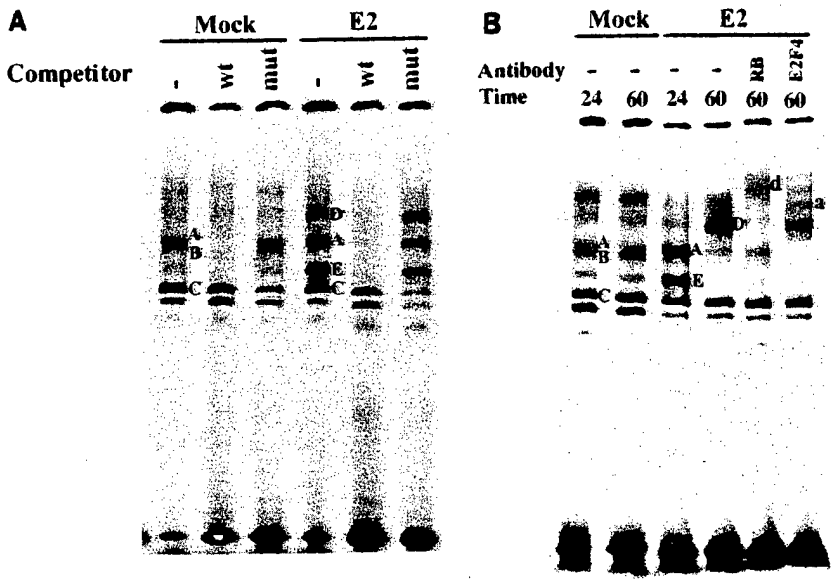


FIG. 6. (A) E2F DNA binding complexes in HT-3 cells. Nuclear extracts were prepared 48 h after mock infection or after infection with a virus expressing the wild-type E2 protein, as indicated. Extracts were incubated with radiolabeled wild-type E2F site 1 oligonucleotide alone or with probe plus 100-fold excess of the unlabeled wild-type (wt) oligonucleotide or a mutant (mut) one containing the E2F site 1 mutations shown in Fig. 1B. Bound protein complexes were separated by gel electrophoresis and detected by autoradiography. The letters indicate the specific bands referred to in the text. (B) E2F DNA binding complexes at various times after infection. Nuclear extracts were prepared 24 and 60 h after mock infection or after infection with a virus expressing the wild-type E2 protein, as indicated. Extracts were incubated with radiolabeled oligonucleotide containing wild-type E2F site 1. Bound protein complexes were separated by gel electrophoresis and detected by autoradiography. The letters indicate the specific bands referred to in the text. Where indicated, supershift analysis was carried out with antibody recognizing $p105^{Rb}$ or E2F4.

extracts of control and E2-expressing cells was supershifted by the anti-E2F4 antibody, generating band a. Band D, which was prominent only in extracts of E2-expressing cells, was efficiently supershifted by anti-$p105^{Rb}$, generating band d, indicating that it contains $p105^{Rb}$. Supershift analysis of extracts prepared 60 h after infection confirmed that band D, the only prominent complex at this time point, contained $p105^{Rb}$ (Fig. 6B). In addition, band D was eliminated by anti-DP antibody, further indicating that it contained an E2F family member (data not shown). Band D was partially supershifted by anti-E2F4 antibody, suggesting that this band may be a mixture of complexes that contain $p105^{Rb}$ and various E2F family members (Fig. 6B and 7). Bands A, B, and E were not supershifted by antibodies to Rb family members, and none of the prominent bands generated by either set of extracts were supershifted by E2F5, p107, or p130 antibodies (Fig. 7 and data not shown). Although we have not identified the proteins present in complex E, its relatively rapid mobility and the supershift analysis summarized above suggest that it does not contain Rb family members. Furthermore, the disappearance of complexes A and E at later times after infection or after infection at higher MOI indicates that these complexes are not involved in maintenance of cdc25A repression.

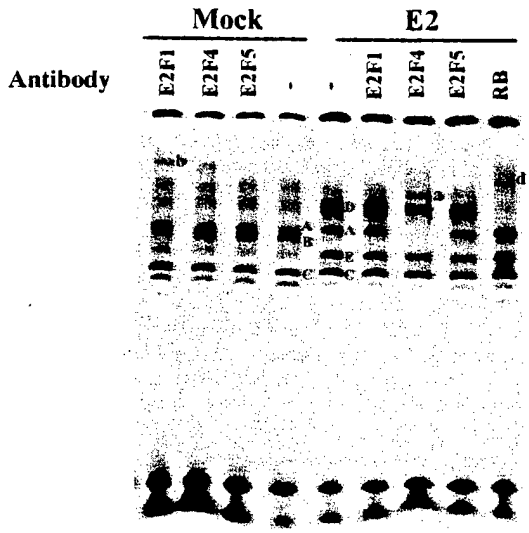


FIG. 7. Identification of protein complexes that bind E2F site 1. Nuclear extracts were prepared as described in the legend to Fig. 6A. Extracts were incubated with wild-type radiolabeled oligonucleotide probe alone (−) or in the presence of the indicated antibody. Bound protein complexes were separated by gel electrophoresis and detected by autoradiography.

We also tested the ability of E2F site 2, which is not required for repression of the cdc25A promoter, to compete for the protein complexes formed at site 1. Extracts prepared 60 h after infection were incubated with radiolabeled oligonucleotide containing site 1 and various amounts of unlabeled oligonucleotides containing site 1 or site 2. As shown in Fig. 8, band D was the predominant retarded species formed on site 1 at this time point, and it was efficiently competed by unlabeled site 1. Site 2 also competed for the formation of band D, but it was a much less effective competitor than site 1. We conclude that $p105^{Rb}$-containing complexes can bind in vitro to either E2F site 1 or site 2, but that the complex binds with higher affinity to site 1, which mediates repression, than to site 2, which does not. Taken together, the results presented in this section strongly suggest that the E2F4-$p105^{Rb}$ complex detected by coimmunoprecipitation is likely responsible at least in part for the appearance of band D and that the binding of this complex or a related complex to E2F site 1 in the cdc25A promoter mediates E2-induced repression of the cdc25A gene.

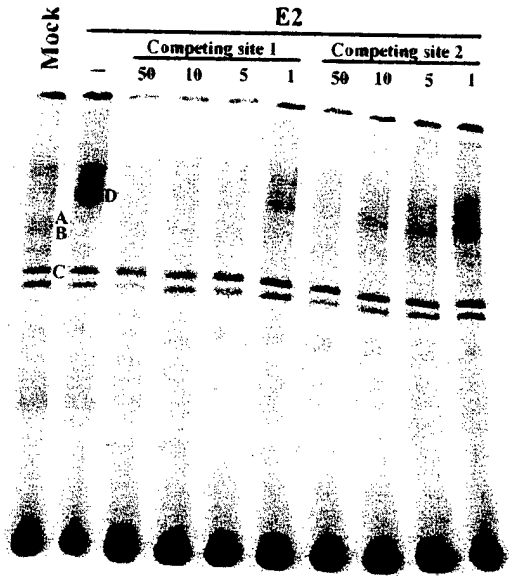


FIG. 8. Competition between E2F site 1 and E2F site 2. Nuclear extracts were prepared at 60 h after mock infection or infection with a virus expressing the wild-type E2 protein. Extracts were incubated with radiolabeled wild-type E2F site 1 oligonucleotide probe alone (−) or in the presence of 1-, 5-, 10-, or 50-fold excess of unlabeled oligonucleotide containing E2F site 1 or E2F site 2, as indicated. Bound protein complexes were separated by gel electrophoresis and detected by autoradiography.

DISCUSSION

Cell cycle regulatory components have been extensively studied in a variety of situations in which cells are induced to enter or exit the cell cycle. However, it is difficult to restore growth control in human cancer cells, because the growth-regulatory pathways in these cells are typically disrupted by mutation. In contrast, the p53 and Rb genes are frequently wild type in cervical carcinoma cells, but their action is masked by the expression of HPV oncogenes. Therefore, these cells represent a unique system to characterize endogenous growth-regulatory pathways in cancer cells, with the hope of identifying pathways that can be manipulated to restore growth control.

To explore the functional consequences of HPV E6/E7 repression on cellular transcription in cervical carcinoma cells, we analyzed the regulation of the cdc25A promoter following repression of E6/E7 expression in HT-3 cells. We first showed that the elements controlling the response of the cdc25A promoter to the E2 protein were contained in a ~500-bp DNA fragment upstream of the translation initiation site. This segment of DNA contains two E2F binding sites, as well as a consensus recognition site for the E2 protein itself. Although an intron of the human cdc25A gene contains an element that binds and responds to Myc and Max transcription factors (18), the fragment conferring E2 responsiveness does not include this element, demonstrating that it was not required for repression in this system. Similarly, our results established that the putative E2 binding site in the cdc25A promoter was not required for E2-mediated repression. Therefore, the cdc25A promoter, unlike the HPV early promoter, is not directly repressed by binding of the E2 protein.

Our results showed that E2-induced repression of the cdc25A promoter was mediated through E2F control. The activity of the cdc25A promoter in proliferating cells was not affected by mutating the E2F sites, ruling out the model that the E2 protein prevented a stimulatory factor from binding to these sites. Instead, mutation of E2F site 1 eliminated E2-mediated repression of the cdc25A promoter, suggesting that the E2 protein induced the expression or activity of an E2F-containing factor that binds to this site and causes repression.

We characterized E2F-containing complexes in HT-3 cells to explore the basis for the E2F site 1-dependent repression of the cdc25A promoter. E2 expression caused marked changes in the abundance of E2F factors and Rb family members. E2F1 levels were depressed, whereas there was increased posttranscriptional accumulation of E2F4, $p105^{Rb}$, and p130. Several mechanisms probably contribute to these effects. In various cell types, high-risk HPV E7 induces rapid degradation of $p105^{Rb}$ via the ubiquitin-proteosome pathway (1, 3, 34). Our results show that this pathway targets $p105^{Rb}$ for rapid degradation in cervical carcinoma cells and suggest that this process can be reversed by repression of HPV E7 expression. In addition, it has been reported that complex formation with Rb family members protects E2F factors from rapid proteosome-mediated degradation (22, 26). The data presented here suggest that induction of endogenous Rb expression in cervical carcinoma cells can result in the stabilization of E2F4 by this mechanism. The abundance of nuclear E2F4-$p105^{Rb}$ and E2F4-p130 complexes increased dramatically as a consequence of the elevated concentrations of the interacting species. In addition, enhanced nuclear localization of E2F4 when it is in complex with Rb family members (37, 38) may also contribute to the accumulation of these complexes in the nucleus.

The E2 protein also caused dramatic changes in the pattern of protein complexes forming at the E2F site responsible for repression of the cdc25A promoter. Complexes containing free E2F1 decreased following E2 expression, whereas complexes containing free E2F4 persisted and even increased in abundance, at least during the initial stages of infection. Most strikingly, the E2 protein induced the appearance of a prominent $p105^{Rb}$-containing complex at the E2F site required for repression. This complex, which appeared to also contain E2F4 or possibly another E2F family member, may also be present in low amounts in proliferating cells, but its abundance increased markedly following E2 expression, and at late times after infection it was the only specific complex formed at site 1 in abundance. This complex was inefficiently competed by site 2, which does not mediate repression. Therefore, we infer that this Rb-containing complex was likely to be responsible for E2-mediated repression. It has recently been shown that E2F site 1 in the cdc25A promoter is required for repression of the cdc25A gene by serum starvation or transforming growth factor β treatment (5, 31, 55), as it is in response to the E2 protein. The importance of this site for negative regulation of the cdc25A gene in these different situations suggests that diverse signals converge on a single common pathway to regulate cdc25A transcription. Transforming growth factor β and alpha interferon treatment also induces the formation of complexes between E2F4 and Rb family members; however, gel shift analysis using the E2F sites from the cdc25A promoter was not carried out, and so it is not known which complexes actually formed at the promoter in response to these treatments (31, 36, 52). Gel shift analysis implied that E2F-p130 complexes forming at E2F site 1 were responsible for repression of the human cdc25A promoter in serum-starved mouse NIH 3T3 cells (5). In contrast to our results, $p105^{Rb}$ complexes were not observed in this heterologous system.

Our results suggest that E2-mediated repression of cell cycle genes in HT-3 cells involves the following sequence of events. By analogy to the HPV18 promoter (45), the E2 protein binds directly to the HPV30 promoter, causing repression of HPV30

E6 and E7 expression. Loss of the E7 protein results in the protection of $p105^{Rb}$ and p130 from rapid proteosome-mediated degradation, leading to increased accumulation of these proteins. $p105^{Rb}$ and p130 then bind to E2F4, protect it from the ubiquitin-proteosome pathway, and promote its relocalization to the nucleus where $p105^{Rb}$-E2F4 and p130-E2F4 complexes accumulate. These $p105^{Rb}$-E2F4 inhibitory complexes bind to E2F site 1 in the cdc25A promoter and impose repression. The E2 protein presumably employs a similar mechanism to repress expression of additional E2F-regulated genes necessary for G_1/S transit, including E2F1 and cyclin A (29, 41, 48, 60; Goodwin and DiMaio, submitted). Thus, once expression of the HPV oncogenes was extinguished, E2F4-Rb complexes characteristic of nonproliferating cells formed and imposed transcriptional control (6, 32, 39, 49, 51). Evidently, the alterations in E2F complexes and in the regulation of E2F-responsive genes that occur during HPV-mediated cervical carcinogenesis are reversible. The integrity of the E2F-Rb axis in cervical carcinoma cells implies that therapeutic interventions that inhibit the activity or expression of HPV oncogenes may lead to a restoration of growth control in these cancers.

ACKNOWLEDGMENTS

We thank Nick Dyson, Frederick Dick, Antonio Iavarone, and Jim DeCaprio for advice and essential reagents, Venkat Reddy for technical assistance, and Janice Zulkeski for assistance in preparing the manuscript.

E.V. was supported by a fellowship from Fondazione Italiana per la Ricerca sal Cancro. This work was supported by grants from the American Cancer Society, National Institutes of Health (CA-16038), and Associazione Italiana per la Ricerca sal Cancro.

REFERENCES

1. **Berezutskaya, E., B. Yu, A. Morozov, P. Raychaudhuri, and S. Bagchi.** 1997. Differential regulation of the pocket domains of the retinoblastoma family proteins by the HPV16 E7 oncoprotein. Cell Growth Differ. **8**:1277–1286.
2. **Bernard, B. A., C. Bailly, M.-C. Lenoir, M. Darmon, F. Thierry, and M. Yaniv.** 1989. The human papillomavirus type 18 (HPV18) E2 gene product is a repressor of the HPV18 regulatory region in human keratinocytes. J. Virol. **63**:4317–4324.
3. **Boyer, S. N., D. E. Wazer, and V. Band.** 1996. E7 protein of human papilloma virus-16 induces degradation of retinoblastoma protein through the ubiquitin-proteosome pathway. Cancer Res. **56**:4620–4624.
4. **Chellappan, S., V. B. Kraus, B. Kroger, K. Münger, P. M. Howley, W. C. Phelps, and J. R. Nevins.** 1992. Adenovirus E1A, simian virus 40 tumor antigen, and human papillomavirus E7 protein share the capacity to disrupt the interaction between the transcription factor E2F and the retinoblastoma gene product. Proc. Natl. Acad. Sci. USA **89**:4549–4553.
5. **Chen, X., and R. Prywes.** 1999. Serum-induced expression of the *cdc25A* gene by relief of E2F-mediated repression. Mol. Cell. Biol. **19**:4695–4702.
6. **Cobrinik, D., P. Whyte, D. S. Peeper, T. Jacks, and R. A. Weinberg.** 1993. Cell cycle-specific association of E2F with the p130 E1A-binding protein. Genes Dev. **7**:2392–2404.
7. **Crook, T., D. Wrede, and K. H. Vousden.** 1991. p53 point mutation in HPV-negative human cervical carcinoma cell lines. Oncogene **6**:873–875.
8. **DeGregori, J., G. Leone, A. Miron, L. Jakoi, and J. R. Nevins.** 1997. Distinct roles for E2F proteins in cell growth control and apoptosis. Proc. Natl. Acad. Sci. USA **94**:7245–7250.
9. **DeGregori, J., T. Kowalik, and J. R. Nevins.** 1995. Cellular targets for activation by the E2F1 transcription factor include DNA synthesis and G_1/S regulatory genes. Mol. Cell. Biol. **15**:4215–4224.
10. **Demers, G., E. Espling, J. Harry, B. Etscheid, and D. Galloway.** 1996. Abrogation of growth arrest signals by human papillomavirus type 16E7 is mediated by sequences required for transformation. J. Virol. **70**:6862–6869.
11. **Demers, G. W., S. A. Foster, C. L. Halbert, and D. A. Galloway.** 1994. Growth arrest by induction of p53 in DNA damaged keratinocytes is bypassed by human papillomavirus 16 E7. Proc. Natl. Acad. Sci. USA **91**:4382–4386.
12. **Desaintes, C., C. Demeret, S. Goyat, M. Yaniv, and F. Thierry.** 1997. Expression of the papillomavirus E2 protein in HeLa cells leads to apoptosis. EMBO J. **16**:504–514.
13. **Dowhanick, J. J., A. A. McBride, and P. M. Howley.** 1995. Suppression of cellular proliferation by the papillomavirus E2 protein. J. Virol. **69**:7791–7799.
14. **Draetta, G., and J. Eckstein.** 1997. Cdc25 protein phosphatases in cell proliferation. Biochim. Biophys. Acta **1332**:M53–M63.
15. **Dyson, N.** 1998. The regulation of E2F by pRB-family proteins. Genes Dev. **12**:2245–2262.
16. **Dyson, N., P. Howley, K. Münger, and E. Harlow.** 1989. The human papillomavirus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. Science **243**:934–936.
17. **Francis, D. A., S. L Schmid, and P. M. Howley.** 2000. Repression of the integrated papillomavirus E6/E7 promoter is required for growth suppression of cervical cancer cells. J. Virol. **74**:2679–2686.
18. **Galaktionov, K., X. Chen, and D. Beach.** 1996. Cdc25 cell-cycle phosphatase as a target of c-*myc*. Nature **382**:511–517.
19. **Ginsberg, D., G. Vairo, T. Chittenden, Z. X. Xiao, G. Xu, K. L. Wydner, J. A. DeCaprio, J. B. Lawrence, and D. M. Livingston.** 1994. E2F-4, a new member of the E2F transcription factor family, interacts with p107. Genes Dev. **8**:2665–2679.
20. **Goodwin, E. C., L. K. Naeger, D. E. Breiding, E. J. Androphy, and D. DiMaio.** 1998. Transactivation-competent bovine papillomavirus E2 protein is specifically required for efficient repression of human papillomavirus oncogene expression and for acute growth inhibition of cervical carcinoma cell lines. J. Virol. **72**:3925–3934.
21. **Hamel, P. A., R. M. Gill, R. A. Phillips, and B. L. Gallie.** 1992. Transcriptional repression of the E2-containing promoters EIIaE, c-*myc*, and RB1 by the product of the RB1 gene. Mol. Cell. Biol. **12**:3431–3438.
22. **Hateboer, G., R. M. Kerkhoven, A. Shvarts, R. Bernards, and R. L. Beijersbergen.** 1996. Degradation of E2F by the ubiquitin-proteasome pathway: regulation by retinoblastoma family proteins and adenovirus transforming proteins. Genes Dev. **10**:2960–2970.
23. **Havre, P. A., J. Yuan, L. Hedrick, K. R. Cho, and P. M. Glazer.** 1995. p53 inactivation by HPV16 E6 results in increased mutagenesis in human cells. Cancer Res. **55**:4420–4424.
24. **Hickman, E., S. Picksley, and K. Vousden.** 1994. Cells expressing HPV16 E7 continue cycle progression following DNA damage-induced p53 activation. Oncogene **9**:2177–2181.
25. **Hiebert, S., S. Chellappan, J. Horowitz, and J. Nevins.** 1992. The interaction of Rb with E2F coincides with an inhibition of the transcriptional activity of E2F. Genes Dev. **6**:177–185.
26. **Hofmann, F., F. Martelli, D. M. Livingston, and Z. Wang.** 1996. The retinoblastoma gene product protects E2F1 from degradation by the ubiquitin-proteasome pathway. Genes Dev. **10**:2949–2959.
27. **Hsiao, K., S. L. McMahon, and P. J. Farnham.** 1994. Multiple DNA elements are required for the growth regulation of the mouse E2F1 promoter. Genes Dev. **8**:1526–1537.
28. **Hurford, R. K. Jr., D. Cobrinik, M. H. Lee, and N. Dyson.** 1997. pRB and p107/p130 are required for the regulated expression of different sets of E2F responsive genes. Genes Dev. **11**:1447–1463.
29. **Hwang, E.-S., L. K. Naeger, and D. DiMaio.** 1996. Activation of the endogenous p53 growth inhibitory pathway in HeLa cervical carcinoma cells by expression of the bovine papillomavirus E2 gene. Oncogene **12**:795–803.
30. **Hwang, E.-S., D. J. Riese II, J. Settleman, L. A. Nilson, J. Honig, S. Flynn, and D. DiMaio.** 1993. Inhibition of cervical carcinoma cell line proliferation by introduction of a bovine papillomavirus regulatory gene. J. Virol. **67**: 3720–3729.
31. **Iavarone, A., and J. Massague.** 1999. E2F and histone deacetylase mediate transforming growth factor β repression of *cdc25A* during keratinocyte cell cycle arrest. Mol. Cell. Biol. **19**:916–922.
32. **Ikeda, M.-A., L. Jakoi, and J. R. Nevins.** 1996. A unique role for the Rb protein in controlling E2F accumulation during cell growth and differentiation. Proc. Natl. Acad. Sci. USA **93**:3215–3220.
33. **Johnson, D. G., K. Ohtani, and J. R. Nevins.** 1994. Autoregulatory control of E2F1 expression in response to positive and negative regulators of cell cycle progression. Genes Dev. **8**:1514–1525.
34. **Jones, D. L., and K. Münger.** 1997. Analysis of the p53-mediated G_1 growth arrest pathway in cells expressing the human papillomavirus type 16 E7 oncoprotein. J. Virol. **71**:2905–2912.
35. **Lam, E. W., and R. J. Watson.** 1993. An E2F-binding site mediates cell-cycle regulated repression of mouse B-myb transcription. EMBO J. **12**:2705–2713.
36. **Li, J.-M., P. P.-C. Hu, X. Shen, Y. Yu, and X.-F. Wang.** 1997. E2F4-RB and E2F4-p107 complexes suppress gene expression by transforming growth factor β through E2F binding sites. Proc. Natl. Acad. Sci. USA **94**:4948–4953.
37. **Lindeman, G. J., S. Gaubatz, D. M. Livingston, and D. Ginsberg.** 1997. The subcellular localization of E2F-4 is cell-cycle dependent. Proc. Natl. Acad. Sci. USA **94**:5095–5100.
38. **Magae, J., C. L. Wu, S. Illenye, E. Harlow, and N. H. Heintz.** 1996. Nuclear localization of DP and E2F transcription factors by heterodimeric partners and retinoblastoma protein family members. J. Cell Sci. **109**:1717–1726.
39. **Moberg, K., M. A. Starz, and J. A. Lees.** 1996. E2F-4 switches from p130 to p107 and pRB in response to cell cycle reentry. Mol. Cell. Biol. **16**:1436–1449.
40. **Morkel, M., J. Wenkel, A. J. Bannister, T. Kouzarides, and C. Hagemeier.** 1997. An E2F-like repressor of transcription. Nature **390**:567–568.
41. **Naeger, L. K., E. C. Goodwin, E.-S. Hwang, R. A. DeFilippis, H. Zhang, and D. DiMaio.** 1999. Bovine papillomavirus E2 protein activates a complex

growth-inhibitory program in p53-negative HT-3 cervical carcinoma cells that includes repression of cyclin A and cdc25A phosphatase genes and accumulation of hypophosphorylated retinoblastoma protein. Cell Growth Differ. 10:413–422.
42. **Neuman, E., E. K. Flemington, W. R. Sellers, and W. G. Kaelin, Jr.** 1994. Transcription of the E2F-1 gene is rendered cell cycle dependent by E2F DNA-binding sites within its promoter. Mol. Cell. Biol. 14:6607–6615.
43. **Nevins, J. R.** 1998. Toward an understanding of the functional complexity of the E2F and retinoblastoma families. Cell Growth Differ. 9:585–593.
44. **Nevins, J. R.** 1992. E2F: a link between the Rb tumor suppressor protein and viral oncoproteins. Science 258:424–429.
45. **Romanczuk, H., F. Thierry, and P. M. Howley.** 1990. Mutations analysis of *cis* elements involved in E2 modulation of human papillomavirus type 16 P_{97} and type 18 P_{105} promoters. J. Virol. 64:2849–2859.
46. **Scheffner, M., K. Münger, J. C. Byrne, and P. M. Howley.** 1991. The state of the p53 and retinoblastoma genes in human cervical carcinoma cell lines. Proc. Natl. Acad. Sci. USA 88:5523–5527.
47. **Scheffner, M., J. M. Huibregtse, R. D. Vierstra, and P. M. Howley.** 1993. The HPV-16 E6 and EP-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. Cell 75:495–505.
48. **Schulze, A., K. Zerfass, D. Spitkovsky, J. Berges, S. Middendorp, P. Jansen-Dürr, and B. Henglein.** 1995. Cell cycle regulation of cyclin A gene transcription is mediated by a variant E2F binding site. Proc. Natl. Acad. Sci. USA 92:11264–11268.
49. **Sidle, A., C. Palaty, P. Dirks, O'N. Wiggan, M. Kiess, R. M. Gill, A. K. Wong, and P. A. Hamel.** 1996. Activity of the retinoblastoma family proteins, pRB, p107, and p130, during cellular proliferation and differentiation. Crit. Rev. Biochem. Mol. Biol. 31:237–271.
50. **Slebos, R., M. Lee, B. Plunkett, T. Kessis, B. Williams, T. Jacks, L. Hedrick, M. Kastan, and K. Cho.** 1994. p53-dependent G1 arrest involves pRB-related proteins and is disrupted by the human papillomavirus 16 E7 oncoprotein. Proc. Natl. Acad. Sci. USA 91:5320–5324.
51. **Smith, E. J., G. Leone, J. DeGregori, L. Jakoi, and J. R. Nevins.** 1996. The accumulation of an E2F-p130 transcriptional repressor distinguishes a G_0 cell state from a G_1 cell state. Mol. Cell. Biol. 16:6965–6976.
52. **Thomas, N. S. B., A. R. Pizzey, S. Tiwari, C. D. Williams, and J. Yang.** 1998. p130, p107, and pRB are differentially regulated in proliferating cells and during cell cycle arrest by α-interferon. J. Biol. Chem. 273:23659–23667.
53. **Tommasi, S., and G. P. Pfeifer.** 1995. In vivo structure of the human *cdc2* promoter: release of a p130–E2F-4 complex from sequences immediately upstream of the transcription initiation site coincides with induction of *cdc2* expression. Mol. Cell. Biol. 15:6901–6913.
54. **Vairo, G., D. M. Livingston, and D. Ginsberg.** 1995. Functional interaction between E2F-4 and p130: evidence for distinct mechanisms underlying growth suppression by different retinoblastoma protein family members. Genes Dev. 9:869–881.
55. **Vigo, E., H. Müller, E. Prosperini, G. Hateboer, P. Cartwright, M. C. Moroni, and K. Helin.** 1999. CDC25A phosphatase is a target of E2F and is required for efficient E2F-induced S phase. Mol. Cell. Biol. 19:6379–6395.
56. **Villa, L. L.** 1997. Human papillomaviruses and cervical cancer. Adv. Cancer Res. 71:321–341.
57. **Weintraub, S. J., K. N. Chow, R. X. Luo, S. H. Zhang, S. He, and D. C. Dean.** 1995. Mechanism of active transcriptional repression by the retinoblastoma protein. Nature 375:812–815.
58. **Weintraub, S. J., C. A. Prater, and D. C. Dean.** 1992. Retinoblastoma protein switches the E2F site from positive to negative element. Nature 358:259–261.
59. **White, A. E., E. M. Livanos, and T. D. Tlsty.** 1994. Differential disruption of genomic integrity and cell cycle regulation in normal human fibroblasts by the HPV oncoprotein. Genes Dev. 8:666–677.
60. **Zerfass, K., A. Schulze, D. Spitkovsky, V. Friedman, B. Henglein, and P. Jansen-Durr.** 1995. Sequential activation of cyclin E and cyclin A gene expression by human papillomavirus type 16 E7 through sequences necessary for transformation. J. Virol. 69:6389–6399.
61. **Zwicker, J., N. Liu, K. Engeland, F. C. Lucibello, and R. Muller.** 1996. Cell cycle regulation of E2F site occupation in vivo. Science 271:1595–1596.